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Transient Expression of Human Adenosine Deaminase cDNAs: Identification of a Nonfunctional Clone Resulting from a Single Amino Acid Substitution

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Human adenosine deaminase (ADA) is an important purine catabolic enzyme which irreversibly deaminates adenosine and deoxyadenosine. Severe genetic deficiency of ADA leads to an immunological deficiency state in which T-lymphoid cells are selectively destroyed by the accumulation of toxic levels of deoxyadenosine and deoxy-ATP. In preparation for transfer of ADA sequences into a variety of cell types, we explored expression of ADA cDNAs transfected into cultured cells within a simian virus 40-based expression vector. After transfection into monkey kidney (COS) cells, ADA cDNA encompassing the entire coding region of the protein generated human ADA activity. An unexpected finding, however, was the identification of a cDNA clone that failed to produce either human enzyme activity or immunoreactive ADA protein. As this pattern is typical of many naturally occurring mutant ADA alleles, we characterized the molecular defect in this clone. DNA sequence analysis revealed a single nucleotide substitution in amino acid position 50 (glycine-valine). Northern blotting with a unique 17-mer oligonucleotide demonstrated the absence of the mutant sequence in the mRNA from which the cDNA library giving rise to the mutant cDNA was constructed. Therefore, the substitution in the variant cDNA was created during cloning. These data define one critical region of the human ADA protein molecule and suggest a convenient strategy for characterization of the phenotypes associated with naturally occurring mutant alleles.

Human adenosine deaminase (ADA) is a purine catabolic enzyme that catalyzes the irreversible deamination of adenosine and deoxyadenosine. Although widely distributed in human tissues, its activity is highest in lymphoid cells, particularly of the T-cell variety (21, 37). The enzyme is active as a monomeric protein of molecular weight 40,000 (6, 8, 39).

Severe genetic deficiency of ADA is associated with an immunological deficiency state known as severe combined immunodeficiency in which immature T-lymphocytes are destroyed due to accumulation of deoxyadenosine and dATP (12, 29). Although the deficiency is genetically heterogeneous, most patients have virtually no detectable enzyme activity and variable, or little, immunoreactive protein in their erythrocytes and residual lymphocytes (3, 5, 7, 16, 33, 40). These findings suggest that severe ADA deficiency arises from structural mutations affecting enzyme activity and stability. Consistent with these conclusions, normal or elevated ADA mRNA levels have been noted in some B-lymphoblast cell lines established from affected patients (1, 8, 36, 38). In some cases, structurally abnormal ADA mRNAs are present (2).

Severe ADA deficiency represents a model of a genetic disorder whose effects are restricted to marrow-derived cells (4). Transfer of ADA gene sequences into marrow stem cells might ultimately provide a means for correction of this disorder. Within the past year, several groups, including our own (8, 26, 35, 38), have isolated cDNA clones encoding ADA. The nucleotide sequences of these cDNAs provide the complete amino acid sequence of the protein (8, 39).

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In preparation for transfer of ADA sequences into diverse cell types in a variety of recombinant vectors, we explored the function of ADA cDNA transfected into cultured cells within a simian virus 40 (SV40)-based expression vector. In the course of experiments leading to the construction of a full-length expressible ADA cDNA, we encountered a cDNA insert that was unable to encode functional or stable ADA protein. In effect, this cDNA mimicked the phenotype of naturally occurring severe ADA deficiency. Location of a single nucleotide substitution that altered a codon of the ADA protein in this cDNA clone points to a potentially critical region of the ADA molecule.

MATERIALS AND METHODS

Reconstruction of ADA cDNA inserts containing the entire coding region. Human ADA cDNA clones spanning the ADA mRNA have been previously isolated and reported (8, 26; Fig. 1). Initially, cDNA clones A3 and C10 were digested with *Bgl*II and religated, and a cDNA containing the 5′ segment derived from A3 and 3′ segment from C10 was isolated. An *Hin*dIII linker was placed at the *Nco*I site in the 3′ untranslated region, and a fragment encompassing the unique *Bam*HI site of the cDNA through this *Hin*dIII linker was cloned into the polylinker segment of plasmid SP64 (24). An *Eco*RI linker was placed at the *Nae*I site within the 5′ untranslated region of cDNA clone E3, and the *Nae*I-*Bam*HI portion of the 5′ end of the cDNA was introduced such that the entire cDNA was represented from an *Eco*RI linker at the *Nae*I site through the *Hin*dIII linker at the *Nco*I site in SP64. An *Eco*RI-*Acc*I digest was performed, and the termini of the 1.3-kilobase (kb) ADA cDNA fragment converted to *Hin*dIII sites. The resulting *Hin*dIII fragment was cloned into the

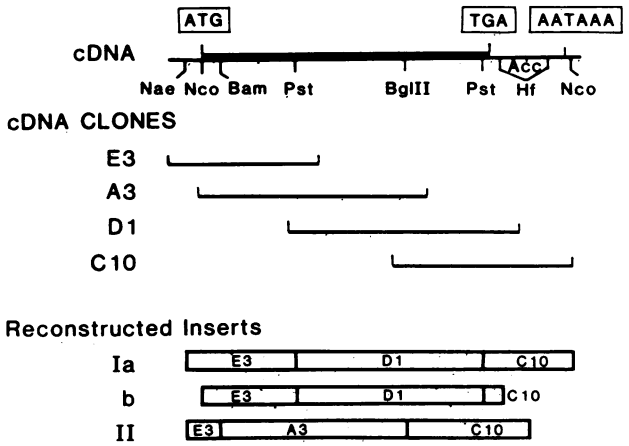

FIG. 1. Reconstructed ADA cDNA inserts. At the top a restriction map of full-length ADA cDNA, deduced from DNA sequences (8), is displayed with the initiator and termination codons and the cleavage-polyadenylation signal enclosed in boxes. The regions of the ADA mRNA sequence spanned by four cDNA clones are shown in the middle section. The cDNA clones from which segments of the reconstructed inserts were derived are shown below.

SV40-based expression plasmid pSV2 (25, 32), in which the unique *Bgl*II site was converted to an *Hin*dIII site. The modified pSV2 plasmid thereby contained a single *Hin*dIII site into which *Hin*dIII-linkered cDNA fragments could be introduced for functional analysis.

Subsequent to construction of pSV2 ADA (II), the inserts designated Ia and Ib (Fig. 1) were assembled. The SP64 plasmid containing the *Eco*RI-*Hin*dIII fragment (*Nae*I through the 3′ *Nco*I site of the cDNA) of insert II was digested with *Pst*I and religated. A clone in which the central *Pst*I fragment of the cDNA was lacking was isolated. This clone was then digested with *Eco*RI and *Pst*I and ligated to the *Nae*I-*Pst*I fragment of E3 in which the *Nae*I site was converted to an *Eco*RI site. The resulting plasmid thereby contained the *Nae*I-*Pst*I region of clone E3 and the *Pst*I-3′ *Nco*I region of clone C10. An isolated 0.65-kb *Pst*I fragment of clone D1 was introduced into the unique *Pst*I site of this construct. A clone was chosen in which the orientation of the internal *Pst*I was correct. The *Eco*RI site at the extreme 5′ end was again converted to an *Hin*dIII site, and the entire insert Ia was placed in pSV2. A 1.1-kb *Nco*I-*Hin*fI fragment of this insert was isolated and cloned with *Hin*dIII linkers directly into pSV2 to generate insert Ib.

All plasmid constructions and analyses were performed by standard procedures as previously described (20). DNA sequencing was performed by the method of Maxam and Gilbert (22), using the strategy previously described (8). The normal human ADA cDNA sequence we have described was assembled from the sequences of clones E3, D1, and C10 (8).

Transient expression of pSV2 ADA constructs. Monkey kidney COS-7 cells (13) were cultured in Dulbecco modified Eagle medium with 10% fetal calf serum. Subconfluent plates were transfected with 20 μg of plasmid DNA per 100-mm dish by calcium phosphate precipitation (34). After 48 h, cells were harvested. ADA isozyme analysis, which distinguishes human and monkey kidney enzymes, was performed by either starch (31) or acrylamide (18) gel electrophoresis and in situ staining (31). Total cell RNA was prepared by guanidine hydrochloride extractions and phenol extraction (27). Polyadenylated poly(A)$^+$ RNA was selected on oligodeoxythymidylic acid cellulose (20).

Western blot analysis. Immunoreactive ADA protein was determined by Western blot analysis as previously described (7) except second antibody, goat anti-rabbit horseradish peroxidase, and 4-chloro-1-napthol were used for color development and detection (15).

Oligonucleotide analysis of a mutant ADA cDNA. A unique 17-mer oligonucleotide of the sequence 5′-TTGTCCAT GACAATGAC-3′ was prepared on a Biosearch synthesizer. This oligonucleotide is complementary to the mRNA strand and spans the altered codon 50 of the mRNA (see Fig. 5 and text). Specificity of the 17-mer probe was first established by dot blot hybridization with pSV2 ADA (Ia and II) clones. Hybridization at 37°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) followed by 6× SSC washing at 37°C and then briefly at 45°C satisfactorily discriminated the sequences. The oligonucleotide was labeled as described previously (28) and hybridized with RNA samples transferred to nitrocellulose (see Fig. 7).

Northern and slot blot analysis. Formaldehyde-treated RNAs were electrophoresed and blotted to nitrocellulose or filtered directly onto filter sheets, using Schleicher & Schuell slot blot apparatus (14, 20). Filters were hybridized with the internal 0.65-kb *Pst*I fragment of ADA cDNA D1 and labeled by the procedure of Feinberg and Vogelstein (11).

RESULTS

Transient expression of functional ADA cDNA. Initial ADA cDNA clones isolated from a cDNA library of human HPB-ALL T-cells did not contain the entire mRNA sequence within a single recombinant (8, 26). Taking advantage of restriction enzyme sites in overlapping cDNA clones, we reconstructed the three cDNA inserts (Ia, Ib, and II) (Fig. 1). Each encompassed the entire coding region, that is, from the initiator ATG through the terminator for translation. Each was derived from three separate clones and contained *Hin*dIII restriction sites at extreme 5′ and 3′ ends (see above). Two inserts (Ia and II) had their 5′ extent at an *Nae*I restriction site approximately 27 nucleotides from the mRNA cap site (unpublished data), whereas insert Ib had the 5′ *Hin*dIII linker added to an *Nco*I site that spanned the initiator ATG. The 3′ extents of the clones differed. Ia had its 3′ *Hin*dIII linker placed at an *Nco*I site 7 nucleotides after the poly(A) addition signal (AATAAA) (30) and 12 nucleotides before the site of polyadenylation. Inserts Ib and II had linkers placed at the 5′-most *Hin*fI and *Acc*I sites, respectively, in the 3′ untranslated region. These latter inserts did not contain the AATAAA sequence in the 3′ untranslated region.

Each insert was recloned in the appropriate orientation within the SV40-based expression vector pSV2 (25, 32), in which a *Bgl*II restriction site was converted to an *Hin*dIII site for convenience (Fig. 2). After transfection into cultured monkey kidney COS cells (13), pSV2 recombinants were amplified, and the cDNA inserts were transcribed from the SV40 early region promoter. Forty-eight hours after transfection, COS cells were harvested and assayed both for ADA enzyme activity by in situ assay in starch gels (31) and polyacrylamide gels (18) as well as quantitative assay (7) and for ADA mRNA sequences by slot blot hybridization and S1 nuclease mapping (10).

COS cells transfected with pSV2 ADA (Ia) displayed abundant human ADA activity (Fig. 3). Human ADA activity was readily separable from monkey kidney enzyme by starch gel electrophoresis. The band of human activity was in general considerably more intense than the endogeneous COS cell activity. In one experiment in which enzyme

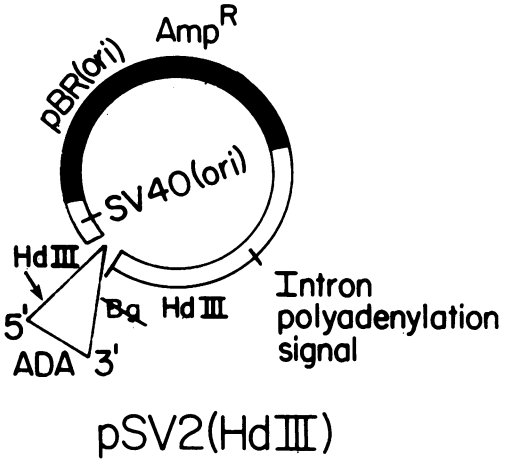

FIG. 2. Structure of the pSV2 (HdIII) transient expression vector. A *Bgl*II restriction site in pSV2 (25, 32) was converted to a HdIII site to facilitate introduction of reconstructed ADA cDNA inserts bearing HdIII linkers at their 5′ and 3′ termini.

activity was quantitated, total ADA activity of transfected cells was 66 nmol/min per mg, representing about sevenfold more than the endogenous activity of COS cells. ADA-specific RNA was abundant in transfected COS cells, as expected (Fig. 3). By Northern blot analysis, ADA-specific RNA derived from insert Ia was about 2.4 kb in length (data not shown), consistent with predominant use of the polyadenylation signal contributed by the pSV2 vector rather than by the ADA insert itself. Transfection of cells with pSV2

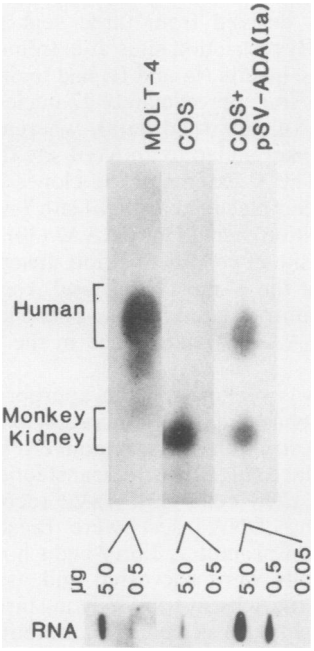

FIG. 3. Expression of human ADA activity and ADA-specific RNA in COS cells transfected with pSV2 ADA (Ia). COS cells were transfected with the pSV2 construct as detailed in the text. Extracts of human MOLT 4, monkey kidney COS, and transfected COS cells were assayed for in situ enzyme activity in starch gel. Total cell RNA in the amounts shown below was treated with formaldehyde and filtered onto nitrocellulose and hybridized with an internal fragment of ADA cDNA (bottom section).

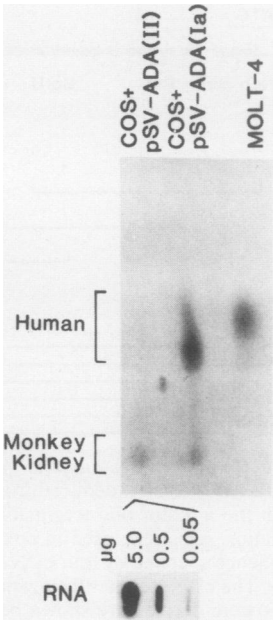

FIG. 4. Failure of COS cells transfected with pSV2 ADA (II) to produce detectable human ADA activity. Transfections and preparations of cell extracts were as described in the text. The slot blot analysis at the bottom demonstrates the presence of abundant ADA-specific RNA in COS cells transfected with pSV2 ADA (II).

ADA (Ib), in which the cleavage-polyadenylation signal (AATAAA) of the ADA cDNA was removed, also led to production of considerable human ADA activity (see below).

Identification of a nonfunctional ADA cDNA insert. In contrast to the above findings, transfection of pSV2 ADA (II) into COS cells did not lead to any detectable human ADA activity by starch gel and activity analyses (Fig. 4). Total ADA activity in extracts of pSV2 ADA (II) was 6.5 nmol/min per mg, a level not appreciably different from that of untransfected COS cells (8.3 nmol/min per mg). The level of ADA-specific RNA sequences was comparable with that obtained with the pSV2 (Ia) and pSV2 (Ib) constructs (Fig. 4). S1 nuclease mapping experiments (data not shown) demonstrated that the mRNA was structurally intact when compared with either T-cell RNA or that generated by the other inserts in COS cells.

The principal difference between insert II and inserts Ia and Ib was the use of ADA cDNA clone A3 rather than D1 (Fig. 1) in assembly of the central portion of the ADA coding sequence. In view of our unexpected results with this clone, the entire insert II was resequenced. In comparison with the normal ADA cDNA sequence (8, 39), a single base change (T to G) was detected (Fig. 5). This substitution predicted substitution of valine for glycine (GGC to GTC) at amino acid 50. Upon realization of this sequence difference, the original insert of ADA cDNA clone A3 was reexamined and also found to contain this substitution (data not shown).

```
                                        50
                          Asn Val Ile Gly Met Asp Lys Pro
Normal cDNA Sequence:     AAC GTC ATT GGC ATG GAC AAG CCG
                                      ▽
Insert II and cDNA A3: AAC GTC ATT GTC ATG GAC AAG CCG
                                   Val
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FIG. 5. Nucleotide substitution identified in codon 50 of the ADA insert II derived from cDNA clone A3.

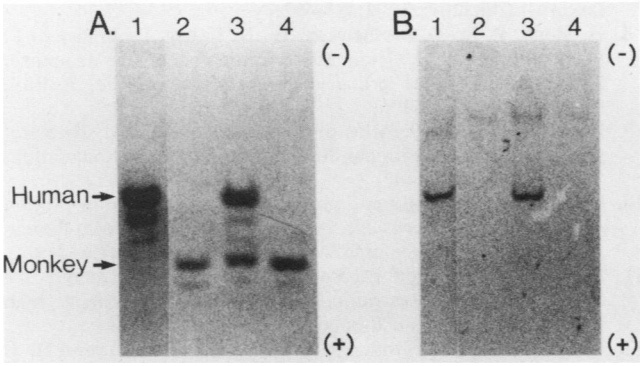

FIG. 6. Enzyme and Western blot analyses of COS cells transfected with pSV2 ADA (Ia) and pSV2 ADA (II). (A) ADA activity strain. Lanes: 1, MOLT 4; 2, COS cells transfected with pSV2 ADA (II); 3, COS cells transfected with pSV2 ADA (Ia); and 4, COS cells. (B) Western blot analysis, as described in the text. Lane designations are the same as in (A). COS cell extracts were diluted to equal protein concentration (85 μg per lane) (19) and incubated with 10 mM iodoacetamide for 5 min to prevent sulfhydryl interactions (17). MOLT 4 extract was diluted to 10 ng of ADA protein (5.5 nmol/min of enzyme activity) per lane and was used as a positive human ADA control.

The absence of ADA activity could have been due either to the presence of a catalytically inactive ADA molecule or to an unstable molecule whose catalytic activity might be normal or decreased. In naturally occurring ADA deficiency the latter is a frequent phenotype. To distinguish between these possibilities, Western blot analysis of lysates of transfected COS cells was performed with heterospecific ADA antibody. COS cells transfected with pSV2 ADA (Ia) showed abundant human ADA activity and immunoreactive protein of about 8 to 9 ng per lane, similar to an extract of human T-MOLT 4 cells (Fig. 6). However, COS cells transfected with pSV2 ADA (II) failed to display any human enzyme activity or any detectable immunoreactive human ADA protein (< 1 ng per lane). These data are most compatible with catabolism of an altered ADA protein within the COS cells.

T to G substitution in the nonfunctional ADA cDNA introduced in cloning. The results obtained with pSV2 ADA (II) posed two alternatives: the T to G substitution might reflect a naturally occurring mutant ADA allele in HPB-ALL cells or the intervention of a cloning artifact, most likely arising during transcription of mRNA by AMV reverse transcriptase. To address these possibilities a unique 17-mer oligonucleotide (5'-TTGTCCATGACAATGAC-3') was prepared for the mutant mRNA sequence and employed in Northern blot hybridization (Fig. 7). Whereas the 17-mer probe detected the mutant ADA mRNA in COS cells after transfection of pSV2 ADA (II), it failed to hybridize with RNA isolated from either HPB-ALL or MOLT 4 T-cells. Hybridization with ADA cDNA probe, however, demonstrated abundant levels of ADA-specific RNA in all samples (Fig. 7). The mutant sequence identified in ADA insert II was not detected in total HPB-ALL RNA and, therefore, arose during cDNA cloning. Alternatively, the mutant RNA sequence might be present in HPB-ALL RNA but at a lower level than that produced from the other (presumably normal) allele. The failure of the oligonucleotide probe to hybridize with HPB-ALL samples in lanes 2 and 4, in spite of their considerably greater ADA mRNA content than the COS sample in lane 1 of Fig. 7, makes this formal possibility unlikely.

DISCUSSION

Transfer of ADA gene sequences into eucaryotic cells represents a potential model for correction of the severe enzyme deficiency that leads to severe combined immunodeficiency. In preparation for introduction of ADA cDNA sequences into a variety of cells, we have constructed nearly full-length ADA cDNA inserts from partial cDNA transcripts. As is to be expected from the experience of others with various cDNAs (32), we have been able to produce considerable human ADA activity upon transfection of cDNA into COS cells in an SV40-based expression vector (Fig. 3, 4, and 6). Even when the AATAAA signal was retained in the ADA cDNA, use of the polyadenylation signal contributed by the SV40 vector predominated. This may be due, in part, to the nature of the sequences located 3' to the cDNA insert, as it is now apparent that downstream sequences affect the efficiency with which putative poly(A) addition sites are utilized (23, 41).

An unexpected finding was the identification of a cDNA insert that failed to generate human ADA activity upon transfection. Careful restriction mapping of this insert excluded trivial explanations for this observation. DNA sequence analysis uncovered a single nucleotide substitution within the clone that altered the predicted protein sequence of ADA at amino acid position 50 (Fig. 5). The replacement of a glycine by a valine is a conservative change. However,

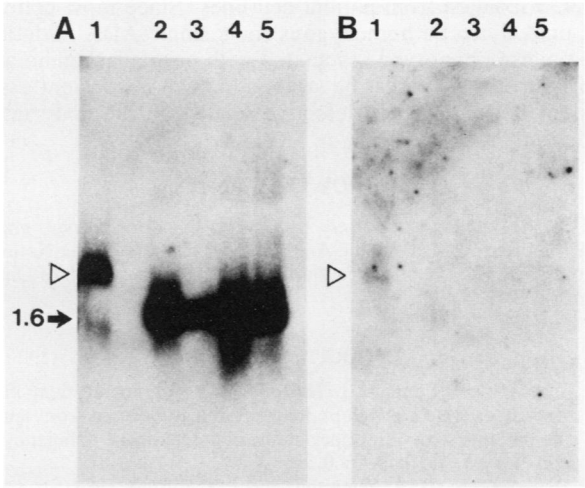

FIG. 7. T to G substitution detected in pSV2 ADA (II) and cDNA clone A3 is not present in HPB-ALL or MOLT 4 T-cell RNA. Poly(A)$^+$ RNAs were formaldehyde treated and electrophoresed in agarose before blot transfer to nitrocellulose (14, 20). The filter was initially hybridized to the unique 17-mer oligonucleotide (5'-TTGTCCATGACAATGAC-3') corresponding to the mutant sequence encompassing codon 50 (B). Hybridization and washing conditions were chosen to maximize discrimination between normal and mutant sequences (see text). After autoradiography the filter was washed in 0.1× SSC at 68°C to remove hybridized oligonucleotide and rehybridized to a radioactive 0.65-kb *Pst*I fragment originating from the central portion of normal ADA cDNA (A). Lanes: 1, 1 μg of poly(A)$^+$ RNA from COS cells transfected with pSV2 ADA (II); 2, 3, and 4, 10, 2, and 5 μg, respectively, of poly(A)$^+$ RNA from HPB-ALL; 5, 10 μg of poly(A)$^+$ RNA of MOLT 4 cells. Only RNA from the COS cells transfected with pSV2 ADA (II) hybridized with the 17-mer probe (open arrow at about 2.3 kb). (A) demonstrates that the level of ADA-specific mRNA in the T-cell samples was equivalent or exceeded that in the sample of COS cell RNA employed. The arrow at 1.6 kb indicates the size of mature T-cell ADA mRNA.

amino acid 50 corresponds precisely with the transition from a beta-strand to a reverse turn or coil in the predicted secondary structure of the protein (8). Therefore, it is likely that the substitution of valine, a hydrophobic branched-chain amino acid, results in loss of peptide flexibility and alteration of the ADA three-dimensional protein conformation (9). Subsequent to this, we envision intracellular catabolism of the aberrant protein. It is not possible to anticipate whether this amino acid substitution alters the active site of ADA, as no stable protein could be detected (Fig. 6). Although we do not consider it likely, the single amino acid substitution might have altered the protein conformation such that the heterospecific ADA-antibody used in the Western blot analysis could not efficiently recognize the protein bound to nitrocellulose (36).

Direct assay with an oligonucleotide (Fig. 7) permits us to conclude that the T to G substitution which led to the effects described above was introduced during cDNA cloning. Although the mutation we observed in an ADA cDNA clone was not present in the RNA of the cells from which the cDNA library was made, our findings remain relevant to the pathophysiology of ADA deficiency. First, the region affected by this mutation (amino acid 50) may prove to be critical in the conformation of the ADA molecule and should be carefully considered upon examination of cDNA clones derived from cells established from affected patients. Second, our results suggest how the transient expression assay in COS cells could be employed to examine putative mutant cDNAs isolated from patient cell lines. Since most patients are unlikely to be homozygous for a single ADA mutation, the precise phenotype associated with particular amino acid replacements may best be studied in such a transient assay system in which each defective allele can be analyzed in isolation.

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